The antioxidant role of thiocyanate in the pathogenesis of cystic fibrosis and other inflammation-related diseases

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Cystic fibrosis (CF) is a pleiotropic disease, originating from mutations in the CF transmembrane conductance regulator (CFTR). Lung injuries inflicted by recurring infection and excessive inflammation cause ≈90% of the morbidity and mortality of CF patients. It remains unclear how CFTR mutations lead to lung illness. Although commonly known as a Cl^- channel, CFTR also conducts thiocyanate (SCN^-) ions, important because, in several ways, they can limit potentially harmful accumulations of hydrogen peroxide (H_2O_2) and hypochlorite (OCl^-). First, lactoperoxidase (LPO) in the airways catalyzes oxidation of SCN^- to tissue-innocuous hypothiocyanite ($OSCN^-$), while consuming H_2O_2. Second, SCN^- even at low concentrations competes effectively with Cl^- for myeloperoxidase (MPO) (which is released by white blood cells), thus limiting OCl^- production by the enzyme. Third, SCN^- can rapidly reduce OCl^- without catalysis. Here, we show that SCN^- and LPO protect a lung cell line from injuries caused by H_2O_2; and that SCN^- protects from OCl^- made by MPO. Of relevance to inflammation in other diseases, we find that in three other tested cell types (arterial endothelial cells, a neuronal cell line, and a pancreatic β cell line) SCN^- at concentrations of ≥100 μM greatly attenuates the cytotoxicity of MPO. Humans naturally derive SCN^- from edible plants, and plasma SCN^- levels of the general population vary from 10 to 140 μM. Our findings raise the possibility that insufficient levels of antioxidant SCN^- provide inadequate protection from OCl^-, thus worsening inflammatory diseases, and predisposing humans to diseases linked to MPO activity, including atherosclerosis, neurodegeneration, and certain cancers.

hydrogen peroxide | hypochlorite | hypothiocyanite | lactoperoxidase | myeloperoxidase

Cystic fibrosis (CF) is the most common fatal hereditary disease in the United States, involving multiple organs, most notably in the respiratory and digestive systems. Lung injuries cause ≈90% of the morbidity and mortality of CF patients. The lungs of CF newborns exhibit no obvious anatomic abnormality, except the presence of somewhat thicker mucus secretion in submucosal glands and possible dilation of the gland ducts (1, 2). Thick secretion may plug the ducts, predisposing them to infection. About 40% of CF infants begin to experience lung infection within 6 months after birth (3). A characteristic of cystic fibrosis is exaggerated inflammation in response to infection (4). In some cases inflammation may happen first (5). Lung injuries prominently occur during infection and inflammation, and may continue at a slower pace even when infection is clinically under control. The injuries in turn make the lungs more susceptible to infection (6), and the very thick purulent mucus resulting from infection and inflammation further aggravates the infection. This vicious cycle compounds the increasingly severe structural alterations and functional deterioration of the lungs, eventually resulting in death.

Twenty-six years ago, epithelial cell membranes of CF patients were found to lack certain ion channels involved in transmembrane Cl^- flux (7–9). Six years later, the gene defective in CF patients was identified (10–12). It encodes an integral membrane protein termed the CF transmembrane conductance regulator (CFTR), which itself is the Cl^--conducting channel. CF-causing mutations generally reduce the number of CFTR channels in cell membranes and/or alter their functionality. However, contrary to the initial expectation that airway Cl^- concentration is altered in CF patients, the Cl^- (and Na^+) concentration in the surface liquid of the CF airways is nearly normal (13, 14). To date, numerous hypotheses have been proposed to account for various aspects of CF pathogenesis. However, the fundamental question remains unanswered: how defective CFTR predisposes CF patients to excessive inflammation entangled with recurring lung infection. CFTR-related defects within the lungs themselves must be the primary cause of the lung illness, and the CF lungs are "proinflammatory." When lungs of non-CF donors are transplanted into CF patients, the lungs do not develop CF-lung illness, as the donor's CFTR gene directs generation of normal CFTR protein in the transplanted lungs. However, when human fetal CF rudiments, containing a defective CFTR gene, are grafted into immune deficient mice, which do not reject grafts, the grafts nonetheless develop progressive, destructive intraluminal inflammation, even before infection. Inflammation makes the grafts prone to infection (15). The importance of inflammation in CF lung illness is underscored by the finding that high doses of the anti-inflammatory agent ibuprofen slow disease progression (16). The lungs of CF patients also experience oxidative stress (17–20), which is an imbalance between the production of reactive oxygen species (ROS) and the ability to effectively detoxify them. A clinical trial on the inhaled antioxidant-glutathione therapy for CF lung disease is ongoing (21).

CFTR channels are known to conduct not only Cl^- ions but also other anions including thiocyanate (SCN^-) (22). SCN^- enters an airway epithelial cell via transporters in its basolateral membrane, and reaches the airway lumen via CFTR in its apical membrane (23). This transepithelial movement results in 460 μM SCN^- in the airway secretions, substantially higher than the plasma concentration (24). SCN^- release is absent in the epithelial cells missing CFTR activity (25, 26). SCN^- reduces certain tissue-damaging species [e.g., hydrogen peroxide (H_2O_2) and hypochlorite (OCl^-)] by subjecting itself to oxidation. H_2O_2 in the normal airways is mostly consumed to produce tissue-innocuous hypothiocyanite ($OSCN^-$) from SCN^-, an oxidation catalyzed by lactoperoxidase (LPO) (Fig. 1*A*) (27). LPO, which is present in both the respiratory and digestive systems, does not catalyze oxidation of Cl^- (28). In the absence of SCN^-, H_2O_2, which has been shown to be harmful to lung epithelial cells (20), should accumulate in the airways. Another important enzyme is myeloperoxidase (MPO), which, unlike LPO, catalyzes two competing reactions: (*i*) H_2O_2 oxidation of Cl^- to highly reactive

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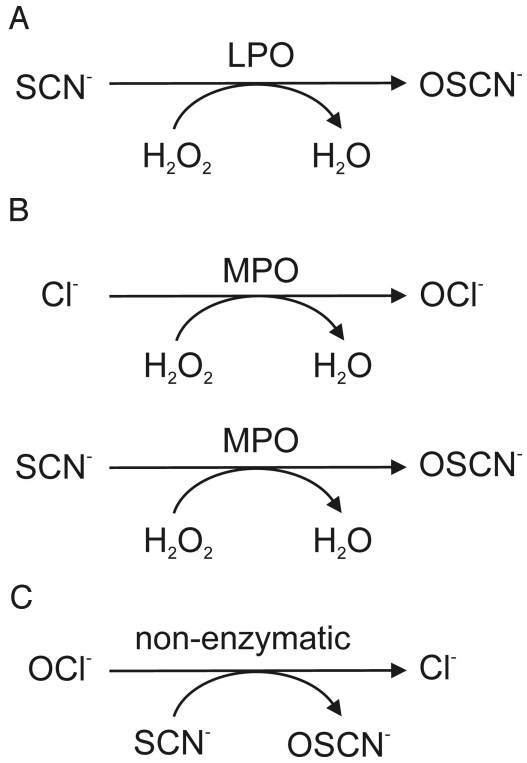

Fig. 1. Lactoperoxidase (LPO) and myeloperoxidase (MPO) catalyzed oxidation reactions. (*A*) LPO catalyzes oxidation of SCN^- to $OSCN^-$ by H_2O_2. (*B*) MPO catalyzes two competitive oxidative reactions: Cl^- to OCl^- (upper) and SCN^- to $OSCN^-$ (lower). (*C*) OCl^- rapidly oxidizes SCN^- to $OSCN^-$ without enzymatic catalysis.

OCl^- (the main active ingredient in household bleach), and (*ii*) oxidation of SCN^- to $OSCN^-$ (Fig. 1*B*) (29). MPO exists almost exclusively in neutrophils, macrophages, and monocytes, which release MPO and H_2O_2 at loci of infection (30). Given that MPO has several hundred-fold lower K_m for SCN^- than for Cl^-, SCN^- may suppress OCl^- production, possibly averting damage to the host. Additionally, SCN^- rapidly scavenges OCl^- without requiring catalysis (Fig. 1*C*) (31). In the absence of adequate SCN^-, overproduction of OCl^- by MPO during inflammation might result in severe lung injuries, and lead to the self-destruction of white blood cells. White cell death would in turn cause additional destructive agents to be dumped, escalating injuries to the host. In the present study, we show that SCN^- prevents MPO from causing injuries to lung cells and that SCN^-, in the presence of LPO, protects the cells against harmful H_2O_2.

Results

SCN^- Protects Cells Against Injuries Caused by MPO Activity. We first used a human lung epithelial cell line, Calu-3, to confirm that MPO causes severe cell injuries or cell death by producing OCl^-. This cell line has been commonly used as a model in CF studies, as it resembles serous cells in the submucosal gland (32). Severely injured or nonviable cells were identified using the standard trypan-blue exclusion method. About 10% of the Calu-3 cells were stained by trypan blue in our control experiments (Fig. 2). As the H_2O_2 concentration in the exhaled air condensate from CF patients with infection may reach the micromolar range (19), we produced a similar concentration of H_2O_2 by using glucose oxidase (GO, 10 mU/mL) to generate H_2O_2 at a rate of $\approx$1 μM/min (20). Under this condition (GO, Fig. 2), H_2O_2 barely increased the percentage of stained cells above background level. Similarly, MPO (1 U/mL) in the absence

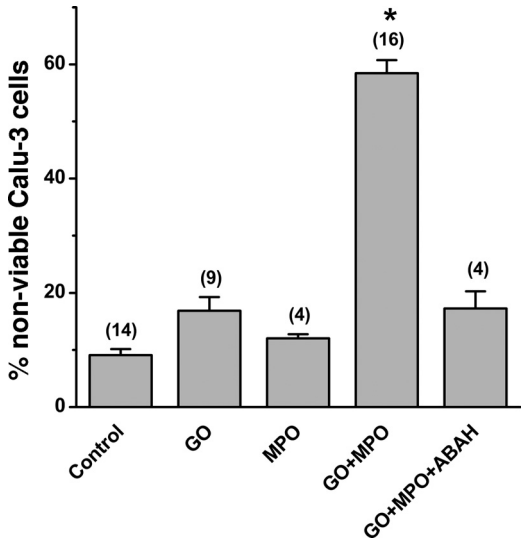

Fig. 2. Cytotoxicity of MPO. Calu-3 cells were incubated in EBSS containing: no added reagents (control); GO; MPO; GO plus MPO; or GO plus MPO with the inhibitor ABAH (GO, 10 mU/mL; MPO, 1 U/mL; ABAH, 100 μM). Percentages of nonviable cells are presented as mean $\pm$ SEM (the number of independent trials is indicated in parentheses). The differences between data with and without asterisk are statistically significant (one-way ANOVA; $P < 0.001$).

of GO had no significant effect on survival (MPO, Fig. 2). However, a combination of GO (10 mU/mL) and MPO (1 U/mL) increased the proportion of nonviable cells to $\approx$60% (GO+MPO, Fig. 2). This increase (in nonviable cells) was prevented by 100 μM 4-aminobenzoic acid hydrazide (ABAH), an MPO inhibitor (20) (GO+MPO+ABAH, Fig. 2). These results confirm previously reported lung cell injuries caused by MPO toxicity (20).

We next performed the crucial test of whether SCN^- could protect cells against MPO toxicity. Fig. 3 shows that 10 μM SCN^- afforded little protection, 50 μM SCN^- provided modest

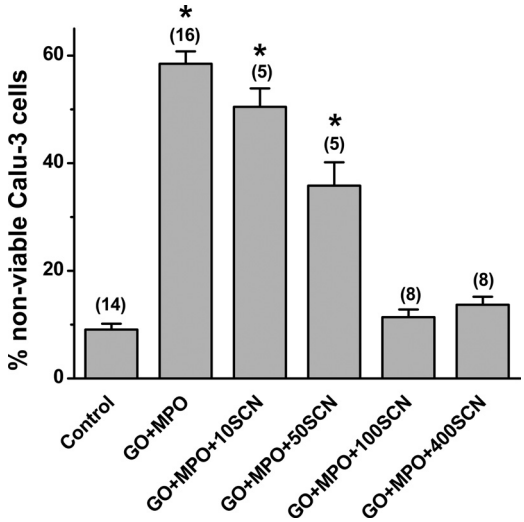

Fig. 3. SCN^- protects Calu-3 cells against MPO cytotoxicity. Cells were incubated in EBSS solution containing: no added reagents (control); GO plus MPO; or GO plus MPO and SCN^- (GO, 10 mU/mL; MPO, 1 U/mL; SCN^- at 10, 50, 100, or 400 μM). Percentages of nonviable cells are presented as mean $\pm$ SEM (the number of independent trials is indicated in parentheses). The differences between data with and without asterisk are statistically significant (one-way ANOVA; $P < 0.001$).

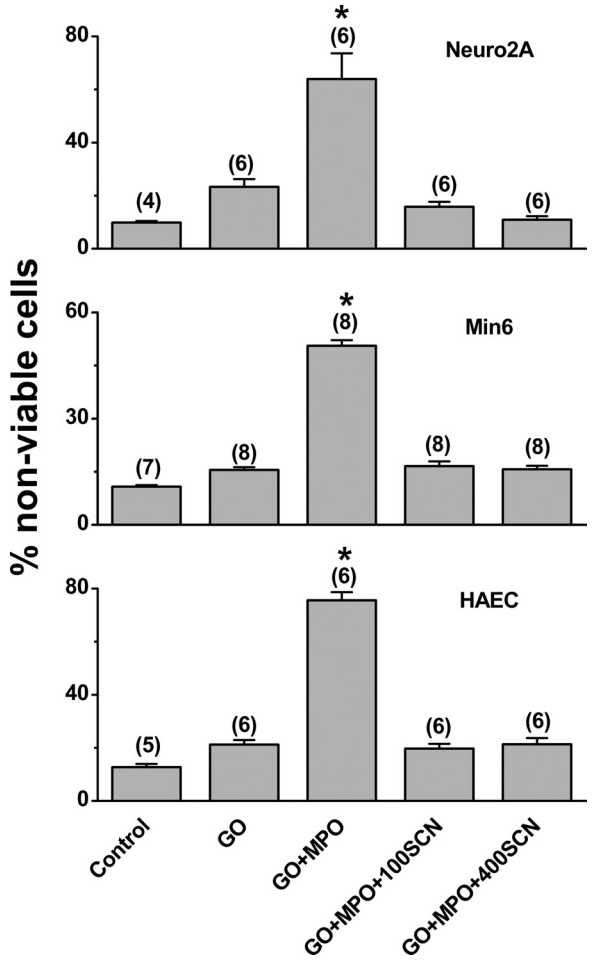

Fig. 4. SCN^- protects Neuro-2A, Min6, and HAE cells against MPO cytotoxicity. Cells were incubated in EBSS containing: no added reagents (control), GO; GO plus MPO; GO plus MPO and SCN^- (SCN^-, 100 or 400 μM; MPO, 1 U/mL; GO, 10 mU/mL for Neuro-2A and Min6 cells and 5 mU/mL for HEA cells). Percentages of nonviable cells are presented as mean ± SEM (the number of independent trials is indicated in parentheses). The differences between data with and without asterisk are statistically significant (one-way ANOVA; $P < 0.001$).

protection, and 100 or 400 μM protected almost fully. Furthermore, we tested the MPO susceptibility of three other cell types: a mouse neuroblastoma cell line (Neuro2a), a mouse pancreatic β cell line (Min6), and human aortic endothelial cells (HAEC). MPO activity rendered 55–75% of these cells nonviable, but all three cell types were virtually fully protected by 100 or 400 μM SCN^- (Fig. 4). The implications of these results are discussed below.

SCN^- Inhibits OCl^- Production by MPO. To corroborate our cell viability assay, we biochemically assayed whether SCN^- could indeed inhibit the MPO-catalyzed production of OCl^-. Fig. 5 plots the concentrations of OCl^- and $OSCN^-$ produced by 1 U/mL MPO in 5 min in the presence of various concentrations of SCN^- and constant 100 mM Cl^-. Increasing SCN^- concentration boosted the production of $OSCN^-$ but depressed that of OCl^-. Consistent with the result of our cell-viability assay, 10 μM SCN^- inhibited only slightly, if at all, the production of OCl^-; 50 μM SCN^- partially inhibited; and 100–400 μM inhibited almost completely. In high concentrations SCN^- strongly suppresses OCl^- production by competing with Cl^- for MPO. Any OCl^- produced would be

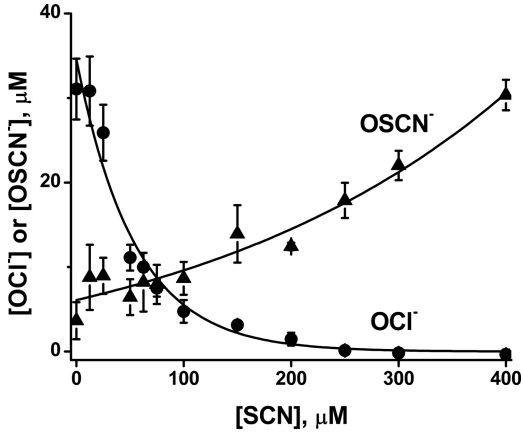

Fig. 5. Production of OCl^- and $OSCN^-$ catalyzed by MPO. Concentrations of OCl^- (●) and $OSCN^-$ (▲) produced in 5 min by MPO (1 U/mL) plotted against the SCN^- concentration in the presence of constant 100 mM Cl^- (mean ± SEM, $n = 4$).

chemically reduced rapidly by SCN^-, via the nonenzymatic reaction diagramed in Fig. 1*C* (31).

LPO and SCN^- Together Prevent Cell Injury Caused by H_2O_2. H_2O_2 by itself causes injuries to airway epithelial cells (20). Some protection may be provided by the LPO-catalyzed reaction shown in Fig. 1*A*, where H_2O_2 is consumed to oxidize SCN^-. To test this possibility we used 20 mU/mL GO to generate enough H_2O_2 to raise the proportion of nonviable cells to ≈40% (Fig. 6). Neither SCN^- (100 or 400 μM) nor LPO (1 U/mL) alone provided any significant protection (Fig. 6). LPO (1 U/mL) plus 100 μM SCN^- partially protected the cells against H_2O_2, and with 400 μM SCN^-, there was essentially full protection (Fig. 6) [the reported SCN^- concentration in the airway fluid is 460 μM (24)].

Discussion

Both the airways and the digestive tract possess the LPO system, which consists of LPO, SCN^-, and H_2O_2-generating enzymes

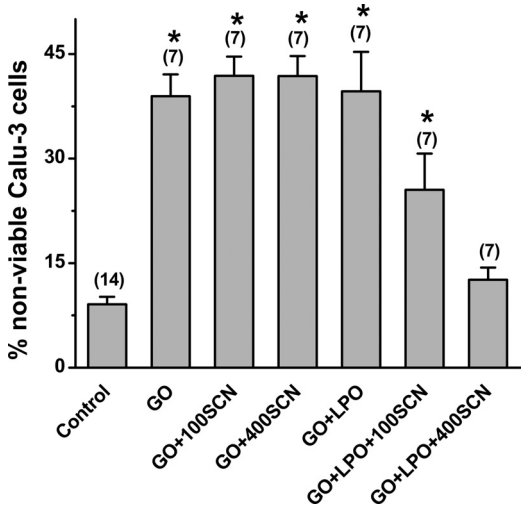

Fig. 6. LPO and SCN^- together prevent H_2O_2 cytotoxicity. Calu-3 cells were incubated in EBSS containing: no added reagents (control), GO; GO plus SCN^-; GO plus LPO; GO plus LPO and SCN^- (GO, 20 mU/mL; LPO, 1 U/mL; SCN^-, 100 or 400 μM). Percentages of nonviable cells are presented as mean ± SEM (the number of independent trials is indicated in parentheses). The differences between data with and without asterisk are statistically significant (one-way ANOVA; $P < 0.001$).

(33). In airways LPO catalyzes oxidation, by H_2O_2, of SCN^- to $OSCN^-$ as it consumes H_2O_2 (Fig. 1*A*). This reaction guards against excessive accumulation of useful but potentially harmful H_2O_2, while at the same time producing antimicrobial $OSCN^-$ (24–26). For the reaction to go forward an adequate supply of SCN^- must be delivered through CFTR channels to the apical surface. Serous cells of submucosal glands not only produce LPO (24), but also express the highest level of CFTR within the lungs (34, 35). Cells along the distal secreting ducts of the glands generate the H_2O_2-producing enzyme dual oxidase (Duox) (36). This is probably why the initial histological abnormality of CF fetuses occurs in submucosal glands (1, 2), because in the absence of SCN^- from the serous cells, unconsumed H_2O_2 would be expected to irritate and harm the secreting glands, leading to inflammation and injuries. Duox activity indirectly stimulates mucin expression by epithelial cells (37); and mucin has a protective effect in that it scavenges ROS, including H_2O_2 (38). The tissue-harming effects of H_2O_2 may contribute to the inflammation observed in the digestive system and the lungs of CF patients in the absence of clinical infection. Here, we find that H_2O_2-caused injuries to the Calu-3 lung cell line can be averted by SCN^- in the presence of LPO (Fig. 6).

Unlike LPO, MPO catalyzes two competing oxidative reactions: Cl^- to highly reactive OCl^- and SCN^- to tissue-innocuous $OSCN^-$. For this reason, the "resting" activity of MPO in the absence of an adequate SCN^- supply may contribute to the pathology in the digestive system and the lungs of CF patients in the absence of clinical infection. However, once infection occurs it is clear that MPO becomes heavily involved. A massive number of neutrophils and macrophages are attracted to infection sites, releasing MPO and H_2O_2 (30) along with other bactericidal and inflammatory agents. MPO activity is known to inflict severe injuries on airway epithelial cells (20), as well as to stop ciliary beating (H_2O_2 is also known to inhibit ciliary beating) (39, 40). Cilia normally propel pathogens out of the airways, and their impairment would allow bacteria to remain and develop infection (41, 42). Here, we find that SCN^- protects cells from MPO-caused injuries (Figs. 3 and 4) in part by inhibiting the production of OCl^- (Fig. 1*B*). Also, SCN^- causes the chemical reduction of any OCl^- produced (Fig. 1*C*): SCN^- is rapidly oxidized by OCl^- to $OSCN^-$ via a nonenzymatic reaction with a second-order rate constant of $2.3 \times 10^7\ M^{-1}\ s^{-1}$ (31). Together, these two actions of SCN^- not only limit the generation of OCl^- but also shorten the lifetime of any OCl^- produced. Thus, inadequate delivery of SCN^- to the airways in CF patients could help explain the excessive damage caused by MPO during inflammation.

In CF patients, there is a high incidence of diabetes partly caused by damage to the pancreatic β cells (43, 44). Interestingly, type 2 diabetes is associated with higher levels of MPO (45). We find that the MPO-caused injuries to a pancreatic β cell line (Min 6) and blood vessel endothelial cells (HAEC) can be greatly reduced, by as little as 100 μM SCN^- (Fig. 4). This finding raises the possibility that MPO, in the absence of adequate SCN^-, contributes to diabetes.

SCN^- is a natural, effective, antioxidant. Given that humans primarily derive SCN^- from vegetables, is it possible that dietary SCN^- deficiency underlies some health problems in a fraction of the general population? Previously reported plasma SCN^- concentrations of the general population range from 10 to 140 μM (46–48). In our experiment, SCN^- at concentrations below 100 μM does not eliminate OCl^- and thus does not fully protect cells against MPO cytotoxicity (Figs. 3 and 4). Conceivably, inadequate SCN^- levels would aggravate MPO-produced injuries in patients suffering from inflammatory diseases including asthma. MPO activity has been linked to lung cancers among smokers (49) and also implicated in the pathogenesis of many neurodegenerative diseases (50–52). We find that MPO-caused injuries to a neuronal cell line (Neuro-2A) can be greatly reduced by SCN^- (Fig. 4). Also, people with congenital MPO deficiency are less likely to develop cardiovascular diseases (53). Conversely, individuals with blood MPO levels in the highest quartile are expected to have a 15- to 20-fold higher chance of coronary artery stenosis, compared with those in the lowest quartile (54, 55). MPO is a critical atherogenic factor (54), and causes endothelial cell death, which is probably involved in the superficial arterial wall erosion that precipitates thrombus formation (56). Here, we show that 100 μM SCN^- largely protects endothelial cells from the injuries caused by MPO activity (Fig. 4). As to the LPO system, it is present in many tissue types (including the lungs and breasts) that contain exocrine glands, and an adequate SCN^- concentration is needed to prevent chronic irritation of these tissues by accumulated H_2O_2 and resulting pathologies.

In summary, genetic defects in CFTR cause malfunction of the digestive system in CF patients and predispose their lungs to excessive inflammation entangled with recurring lung infection. Defective CFTR channels would be expected to result in lower SCN^- concentrations in the affected regions within the respiratory and digestive systems, leaving tissues inadequately protected from accumulated H_2O_2 and overproduced OCl^-. Here, we find that SCN^-, in the presence of LPO, protects cells from injuries by H_2O_2, and that it also prevents MPO-caused cell injuries by suppressing production of OCl^- and speeding its reduction. Conceptually, delivering SCN^- directly to the digestive and respiratory systems might be a therapy for CF disease. SCN^- supply to the affected regions may also be increased by raising plasma SCN^- concentration to boost SCN^- efflux through residual functional CFTR channels and/or through other non-CFTR anion channels or transporters. (If serum SCN^- is raised, adequate iodine must be provided because SCN^-, a known goitrogen, inhibits iodine uptake.) Additionally, proper suppression of the harmful ROS-generating activity of Duox and/or MPO with specific exogenous compounds should be explored. As to the general population, individuals with low plasma SCN^- concentrations may be at risk for chronic insidious injuries by MPO, predisposing them to inflammatory (or inflammation-mediated) diseases. Many have proposed to develop drugs that specifically inhibit MPO-catalyzed OCl^- production to combat these diseases (57). Fortunately, evolution has provided SCN^-, which not only decreases MPO-catalyzed formation of OCl^- but also rapidly scavenges it.

Materials and Methods

Cell Viability Assay. Four types of cells, Calu-3, Min6 (58), Neuro-2A, and HAEC (Invitrogen Inc.) were plated in 48-well culture plates (3.5×10^4 to 2×10^5 per well). Twenty-four to forty-eight hours later, cells were washed with Earl's balanced salt solution (EBSS) before subsequent studies. EBSS contained: NaCl (116 mM), glucose (5.5 mM), KCl (5.4 mM), $CaCl_2$ (1.8 mM), $MgSO_4$ (2.2 mM), NaH_2PO_4 (1 mM), and $NaHCO_3$ (26 mM); pH 7.0. To assess the possible protective effect of LPO and SCN^- on H_2O_2 cytotoxicity, Calu-3 cells were incubated for 3 h in EBBS containing GO (20 mU/mL), while maintained in a 37°C incubator (5% CO_2). LPO (1 U/mL) and NaSCN (100 or 400 μM) were added to protect cells from injuries caused by H_2O_2. For the MPO studies, cells were first incubated for 1 h in EBSS containing MPO (1 U/mL). The MPO reaction was initiated by adding 10 mU/mL GO to generate H_2O_2, and cells were maintained in a 37°C incubator (5% CO_2) for 3 additional hours. NaSCN (10–400 μM) or the MPO inhibitor ABAH (100 μM) were added, before the addition of GO, to protect cells from injuries caused by MPO activity. Because HAE cells were more sensitive to reactive oxygen species, GO was reduced to 5 mU/mL and the incubation period to 1 h. After incubation, cells were trypsinized, pelleted, and resuspended in a phosphate buffer solution (PBS), which contains NaCl (137 mM), KCl (2.7 mM), Na_2HPO_4 (4.3 mM), and KH_2PO_4 (1.4 mM); pH 7.3. Nonviable cells were identified by staining with 0.2% Trypan blue (Invitrogen, Inc.) (59). Both stained and unstained cells were counted with a hemocytometer in double blind experiments.

Biochemical Assay of MPO-Catalyzed ClO^- and $OSCN^-$ Production. Reaction mixtures contained MPO (1 U/ mL), NaCl (100 mM), NaSCN (0–400 μM), and sodium phosphate (100 mM, pH 7.0). Reactions were initiated by adding 50 μM H_2O_2 to the mixture, and stopped 5 min later by adding 1 μM catalase to decompose remaining H_2O_2 to H_2O and O_2. Blank reactions contained no H_2O_2. The concentrations of OCl^- and $OSCN^-$ were determined using the taurine chloramine assay (60). OCl^- and $OSCN^-$ react with taurine to form taurine chloramine. The latter oxidizes yellow 5-thio-2-nitrobenzoic acid (ε_{412} = 14,100 M^{-1} cm^{-1}) to colorless 5,5′-dithiobis-2-nitrobenzoic acid. The drop in absorbance at 412 nm (after subtracting the blank) reflects the total concentration of OCl^- plus $OSCN^-$ produced. The $OSCN^-$ component was isolated by prior addition of 5 mM methionine to reduce OCl^-, which is a strong oxidant; $OSCN^-$ does not readily react with methionine (29). Methionine was added immediately after the addition of catalase but 5 min before the taurine chloramine assay. This 5-min interval allowed methionine to reduce OCl^-. Unless specified otherwise, all reagents were purchased from Sigma-Aldrich.

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